Comparative Biochemistry and Physiology, Part B 154 (2009) 68–79

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

AMP-activated protein kinase and carbohydrate response element binding protein: A study of two potential regulatory factors in the hepatic lipogenic program of broiler chickens ☆

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ARTICLE INFO

Article history:
Received 23 March 2009
Received in revised form 4 May 2009
Accepted 5 May 2009
Available online 8 May 2009

Keywords:
AMP-activated protein kinase
Carbohydrate response element binding protein
Liver
Lipogenesis
Gene expression
Chicken

ABSTRACT

This study investigated the effects of fasting and refeeding on AMP-activated protein kinase (AMPK) and carbohydrate response element binding protein (ChREBP) mRNA, protein and activity levels; as well as the expression of lipogenic genes involved in regulating lipid synthesis in broiler chicken (*Gallus gallus*) liver. Fasting for 24 or 48 h produced significant declines in plasma glucose (at 24 h), insulin and thyroid hormone (T_3) levels that were accompanied by changes in mRNA expression levels of hepatic lipogenic genes. The mRNA levels of malic enzyme (ME), ATP-citrate lyase (ACL), acetyl-CoA carboxylase α (ACCα), fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD-1) and thyroid hormone responsive Spot 14 (Spot 14) declined in response to fasting. Refeeding for 24 h increased mRNA levels for each of these genes, characterized by a significant increase ('overshoot') above fed control values. No change in mRNA expression of the two AMPK alpha subunit genes was observed in response to fasting or refeeding. In contrast, ChREBP and sterol regulatory element binding protein-1 (SREBP-1) mRNA levels decreased during fasting and increased with refeeding. Phosphorylation of AMPK alpha subunits increased modestly after a 48 h fast. However, there was no corresponding change in the phosphorylation of ACC, a major downstream target of AMPK. Protein level and DNA-binding activity of ChREBP increased during fasting and declined upon refeeding as measured in whole liver tissue extracts. In general, evidence was found for coordinate transcriptional regulation of lipogenic program genes in broiler chicken liver, but specific regulatory roles for AMPK and ChREBP in that process remain to be further characterized.

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1. Introduction

In mammals, liver is the principal organ involved in managing long-term energy balance because of its central role in the control of whole-body carbohydrate and lipid metabolism through its ability to store, synthesize and release glucose and its ability to synthesize fatty acids (Dentin et al., 2006). Glucose derived from dietary carbohydrate is processed by the liver to yield energy (ATP) for immediate use or lipid (triglycerides) destined for long-term storage by coordinated regulation of carbon flux through glycolytic and lipogenic metabolic pathways (Dentin et al., 2005; Uyeda and Repa, 2006). Key hepatic enzymes involved in glycolysis and lipogenesis are subject to acute regulation by post-translational modification (e.g., phosphorylation) and allosteric modulation of their activities in response to changing glucose and hormone levels (Uyeda and Repa, 2006). Long-term regulation of these enzymes is achieved through alterations in the level of gene transcription mediated by the actions of specific transcription factors and their coregulators (Dentin et al., 2005; Desvergne et al., 2006).

Insulin and 3,5,3′-triiodothyronine (T_3) have long been recognized to be two important inducers of hepatic lipogenic enzyme gene transcription, with the specific effects of insulin being mediated by sterol regulatory element binding protein-1c (SREBP-1c), a member of the basic helix-loop-helix (bHLH) family of transcription factors (Foufelle and Ferre 2002). There is also now considerable evidence suggesting that glucose itself contributes to the coordinated regulation of carbohydrate and lipid metabolism in the liver (Dentin et al., 2005). Glucose-specific effects on glycolytic and lipogenic gene transcription involve the carbohydrate response element binding protein (ChREBP), which is activated (via dephosphorylation) in response to increased cellular

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1096-4959/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.cbpb.2009.05.003

levels of specific intermediary metabolites of glucose such as glucose-6-phosphate (G6P) or xylulose-5-phosphate (Xu5P) (Dentin et al., 2004; Uyeda and Repa, 2006; Postic et al., 2007). Activated ChREBP binds to carbohydrate response element (ChoRE) sites located within target gene promoter regions, but only in the presence of its coactivator Max-like protein X (Mlx) with which it forms a dimeric complex (Shih et al., 1995; Kawaguchi et al., 2001; Stoeckman et al., 2004, Ma et al., 2005, 2007). In fact, both ChREBP and SREBP-1c have been shown to be key regulators of hepatic glucose metabolism and lipid synthesis (Dentin et al., 2005). Regulated primarily by post-translational mechanisms, SREBP-1c and ChREBP work in concert to increase transcription of glycolytic and lipogenic genes (Dentin et al., 2005; Uyeda and Repa, 2006; Postic et al., 2007).

Additional transcriptional regulatory proteins have been implicated in the control of hepatic glycolytic and lipogenic enzyme gene expression including the small acidic protein Spot 14 (also known as thyroid hormone responsive Spot 14 protein or THRSP) which is highly expressed in lipogenic tissues such as liver and adipose tissue (Liaw and Towle, 1984; Jump and Oppenheimer, 1985; Freak and Oppenhimer, 1987; Jump et al., 1993; Kinlaw et al., 1995; Brown et al., 1997; Cambell et al., 2003). It has been suggested that Spot 14 might act as transcription factor or coregulator for genes involved in lipid synthesis (Compe et al., 2001). In mammals, carbohydrate feeding (glucose) and insulin have been shown to induce hepatic *Spot 14* gene expression via the actions of ChREBP and SREBP-1c, respectively (Shih and Towle, 1992; Koo et al., 2001). Moreover, transcription of *ChREBP*, *SREBP-1c* and *Spot 14* genes is regulated, in part, by the liver X receptor (LXR), another bHLH transcription factor which is expressed in mammals as two isoforms (α and β) with LXRα (also known as NR1H3) being predominately involved in the control of lipogenic gene transcription (Repa et al., 2000; Chen et al., 2004). LXRs act in conjunction with the retinoid X receptor (RXR) to form LXR/RXR heterodimers that bind to LXR response elements (LXREs) to upregulate the expression of *SREBP-1c* and *ChREBP* genes (Chen et al., 2004; Cha and Repa, 2007). Transcription of target lipogenic enzyme genes such as fatty acid synthase (FAS), acetyl-CoA carboxylase α (ACCα), and stearoyl-CoA desaturase-1 (SCD-1) is upregulated by the direct actions of LXR/RXR complexes on LXREs and indirectly by increasing the action of SREBP-1c on SRE elements contained in their promoters (Joseph et al., 2002; Talukdar and Hillgartner, 2006; Denechaud et al., 2008). These findings suggest that complex and highly integrated transcriptional regulatory networks control hepatic lipogeneic gene expression in response to glucose and insulin signaling.

The AMP-activated protein kinase (AMPK) is a highly conserved serine/threonine kinase that functions as an energy sensor and transducer of cellular metabolism (Hardie, 2007). AMPK represents a metabolic checkpoint for the convergence of intracellular signal transduction cascades involved in regulating energy balance by integrating information conveyed by specific nutrient (e.g., glucose and fatty acids) and hormone (e.g., insulin, ghrelin, adiponectin, leptin and glucagon) signals, the levels of which reflect changes in energy status (Kahn et al., 2005). Activation of AMPK requires the phosphorylation of a threonine residue (T172) contained within the alpha catalytic subunit by an upstream kinase such as LKB1 (Hawley et al., 1996; Stein et al., 2000). AMPK has been implicated in the control of hepatic glucose and lipid metabolism, thereby affecting whole body fuel utilization (Viollet et al., 2006, 2009). In short-term regulation, AMPK phosphorylates and inactivates ACCα, thus inhibiting the production of malonyl-CoA and fatty acid biosynthesis (Viollet et al., 2006). In addition, AMPK also phosphorylates and activates malonyl-CoA decarboxylase (MCD) which works to decrease cellular malonyl-CoA. The net effect of these actions is to reduce the level of a potent allosteric inhibitor of carnitine palmitoyl-CoA transferase-1 (CPT-1), a mitochondrial membrane transporter and rate-limiting enzyme controlling fatty acid oxidation (Saggerson, 2008). Thus, by modulating malonyl-CoA levels, AMPK acts as a metabolic regulator shifting the balance of hepatic fuel utilization from glucose to fatty acids while inhibiting *de novo* fatty acid synthesis from glucose. Long-term regulation of glycolytic and lipogenic gene expression in liver results, in part, from decreased DNA binding of *SREBP-1c* and *ChREBP* due to the phosphorylation (i.e., inactivation) of both transcription factors by AMPK (Viollet et al., 2009).

The chicken represents an excellent comparative animal model to study the mechanisms involved in glucose-dependent regulation of hepatic lipid metabolism because of a number of important differences distinguishing birds from mammals including: 1) liver is the predominant site of lipid synthesis in birds (Leveille et al., 1975); 2) blood glucose levels are normally higher in birds, both in the fed and fasted state (Braun and Sweazea, 2008); 3) birds are resistant to the hypoglycemic effects of exogenously administered insulin while exhibiting high sensitivity to glucagon (Hazelwood, 1984; Akiba et al., 1999); 4) insulin signaling pathway activity differs in skeletal muscle as compared to liver in birds (Dupont et al., 2008); 5) hepatic glucose metabolism differs in birds which exhibit low activity of glucokinase (GK) (Rideau et al., 2008), a lack of the liver-type pyruvate kinase (L-PK) isozyme (chickens express the M type isozyme) (Strandholm et al., 1975), and markedly decreased activity of the hexose monophosphate shunt (pentose) pathway (Goodridge, 1968); and 6) birds express a single isoform for SREBP-1 and LXR, and two isoforms (α and β) for Spot 14 (Gondret et al., 2001; Wang et al., 2004; Han et al., 2009). Previous work has identified and characterized avian gene homologues for each of the AMPK subunits, *ChREBP* and its co-activator *Mlx*, *SREBP-1*, *Spot 14* and *LXR*, as well as determined their expression in liver (Gondret et al., 2001; Assaf et al., 2003; Wang et al., 2004; Zhang and Hillgartner, 2004; Proszkowiec-Weglarz et al., 2006a,b, 2008; Zhan et al., 2006; Proszkowiec-Weglarz and Richards, 2007; Han et al., 2009). However, specific roles for each of these regulatory factors in the glucose-dependent regulation of hepatic lipid synthesis in birds have not been fully elucidated. Therefore, the goal of this work was to further characterize the expression and actions of AMPK and ChREBP, as well as some of their downstream target and associated lipogenic genes in broiler chicken liver under altered energy states induced by fasting and refeeding.

2. Materials and methods

2.1. Animals, experimental protocol and sample collection

All animal studies were conducted according to research protocols approved by the Beltsville Animal Care and Use Committee (USDA, ARS). Day-old male broiler (Ross) chicks (*Gallus gallus domesticus*) were purchased from a local hatchery (Moyer's Chicks, Quakertown, PA, USA) and grown until 3 weeks of age. All birds were provided with a standard corn/soy-based poultry diet containing 21% crude protein (Rosebrough et al., 2002) and water *ad libitum*.

Thirty 3-week-old broiler chickens were divided into five equal treatment groups: control, fed *ad libitum* (C); fasted for 24 h (S24), fasted for 24 h and then refed for 24 h (S24R24), fasted for 48 h (S48), and fasted for 48 h and then refed for 24 h (S48R24). At the end of each treatment period chickens were bled by cardiac puncture and the blood (5 mL) was collected into tubes containing EDTA. Plasma was obtained by centrifugation (1000 *g*, 30 min, 4 °C) and stored at −20 °C for further analysis of glucose, insulin, glucagon, leptin, T_3, and thyroxine (T_4). For glucagon analysis, an aliquot of plasma was stored in the presence of 1000 kIU of aprotinin, a protease inhibitor used to prevent its degradation. Liver tissue samples were also collected and most were snap frozen in liquid nitrogen and stored at −80 °C prior to RNA or protein isolation. Selected samples were processed immediately after collection to obtain nuclear protein fractions.

2.2. Reverse transcription polymerase chain reaction (RT–PCR)

Total RNA was isolated from liver tissue samples using the Trizol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad,

CA, USA). Reverse transcription (RT) reactions (20 µL) consisted of: 1.0 µg total RNA, 50 units Superscript III reverse transcriptase (Invitrogen), 40 units of an RNAse inhibitor (Invitrogen), 0.5 mM dNTPs, and 100 ng of random hexamer primers. Polymerase chain reaction (PCR) was performed in 25 µg reactions containing: 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.0 unit of Platinum Taq DNA polymerase (Invitrogen), 0.2 mM dNTPs, 2.0 mM Mg^{2+}, 10 pmol of each gene specific primer (see Table 1 for primer sequences), 5 pmol each of an appropriate mixture of primers and competimers specific for 18 S rRNA (QuantumRNA Universal 18 S Standards kit, Ambion, Inc., Austin, TX, USA), and 1.0 µL of the RT reaction. Thermal cycling parameters were: 1 cycle 94 °C for 2 min, followed by 30 cycles, 94 °C for 30 s, 58 °C or 60 °C for 30 s, 72 °C for 1 min with a final extension at 72 °C for 8 min. Negative PCR controls were run to ensure PCR accuracy and specificity. Gene specific PCR products were isolated using a GenElute PCR clean-up kit (Sigma) and their sequences were verified by bi-directional automated fluorescent DNA sequencing (CEQ 8000XL, Beckman Coulter, Inc., Fullerton, CA, USA).

2.3. Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF)

Relative quantitation of PCR products was accomplished using CE-LIF as described previously (Richards and Poch, 2002). Briefly, aliquots (2 µL) of RT–PCR samples were first diluted 1:100 with deionized water. A P/ACE MDQ CE instrument (Beckman Coulter, Inc.) equipped with an argon ion LIF detector was used. Capillaries were 75 µm I.D. × 32 cm µSil-DNA (Agilent Technologies, Palo Alto, CA, USA). EnhanCE™ dye (Beckman Coulter, Inc.) was added to the DNA separation buffer (0.5%, w/v hydroxypropylmethylcellulose in TBE buffer) to a final concentration of 0.5 µg/mL. Samples were loaded by electrokinetic injection at 3.5 kV for 5 s and run in reverse polarity mode at 8.1 kV for 4.0 min. P/ACE MDQ software (Beckman Coulter, Inc.) was used to calculate peak areas for the individual PCR amplicons.

Table 1
Gene-specific primers used for the analysis of chicken gene expression by RT–PCR.[a]

Gene	GenBank accession number[b]	Primer sequence (5′→3′)	Orientation	Product size (bp)
AMPK α-1	NM_001039603	AAGGTTGGCAAGCATGAGTT	Forward	492
		TTCTGGGCCTGCATATAACC	Reverse	
AMPK α-2	NM_001039605	AGCACGCCAACAGACTTCTT	Forward	399
		ATCATCAAAGGGCAAAGTGC	Reverse	
Mlx	EF426481	CCCAGGAGCACTACAAGGAG	Forward	392
		GGAATCCATAATGCCTTGGA	Reverse	
ChREBP	EU152407	CTGAGCGATCGAAGGTGAA	Forward	483
		TCTCCATCTTGCTGGAGTCA	Reverse	
SREBP-1	AY029224	GAGGAAGGCCATCGAGTACA	Forward	392
		GGAAGACAAAGGCACAGAGG	Reverse	
LXR	AF492498	CCCAAGTCCCTGACCCTAAT	Forward	449
		GGCTTCCACATAGGTGTGCT	Reverse	
Spot 14α	AY568628	ACCGACCTCACCAAAAACAC	Forward	390
		GTGGTCTAGGCACATGCAGA	Reverse	
Spot 14β	AY568630	GATGGAGCAGGAGGTGATGT	Forward	225
		GTCAGGTGGCTGAGGATCTG	Reverse	
ACC	NM_205505	CACTTCGAGGCGAAAAACTC	Forward	447
		GGAGCAAATCCATGACCACT	Reverse	
MCD	XM_414174	GGCCTCACGTCAAGGAAAT	Forward	395
		AAGTTCCACTCCCTGCAGTC	Reverse	
ACL	AJ245664	GGTGACCACAGGCAGAAGTT	Forward	452
		ACCCCTTCATAGACCCCATC	Reverse	
ME	AF408407	ATGAAGAGGGGCTACGAGGT	Forward	470
		CCCATTCCATAACAGCCAAG	Reverse	
FAS	J04485	AAAGGAGATTCCAGCATCGTGCAGC	Forward	423
		GGAGTCAAACTAGTTATCCATGGCC	Reverse	
SCD-1	NM_204890	TCCCTTCTGCAAAGATCCAG	Forward	402
		AGCACAGCAACACCACTGAG	Reverse	

[a] All primers used for mRNA expression analysis were designed using the Primer 3 program (Rozen and Skaletsky, 2000) in the coding region of each gene for which sequence had been previously verified by molecular cloning and direct sequencing of PCR generated fragments.
[b] Reference chicken gene sequences that contain the corresponding PCR products listed.

2.4. Quantification of gene expression

The levels of gene expression were determined as the ratio of integrated peak area for each PCR product relative to that of the co-amplified 18 S rRNA internal standard. Values are presented as the mean ± SEM ($n = 6$) of individual expression ratio determinations.

2.5. Liver protein extraction

To obtain whole tissue lysates, frozen liver tissue was homogenized in T-PER tissue protein extraction reagent (Pierce, Rockford, IL, USA) supplemented with 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1X Halt protease inhibitor cocktail kit (Pierce) and 1X Halt phosphatase inhibitor cocktail (Pierce). Homogenates were then centrifuged at 14,000 *g* for 10 min at 4 °C. The supernatants were collected, snap frozen in liquid nitrogen and stored at −80 °C prior to analysis. Nuclear and cytoplasmic protein fractions were prepared from fresh liver tissue using NE-PER nuclear and cytoplasmic extraction reagents (Pierce). Protein concentration in whole tissue lysates and the nuclear and cytoplasmic fractions was estimated using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Western blot analysis

Whole tissue lysates (50 µg protein) were subjected to SDS polyacrylamide gel electrophoresis under reducing conditions on 10% gels according to the method of Laemmli (1970). Separated proteins were then transferred to PVDF membranes (Immobilon-P, Millipore Corp., Bedford, MA) using a semi-dry electro-blotting system (Bio-Rad Laboratories) for 1.5 h at 16–25 V in Tris-glycine buffer containing 20% methanol. Membranes were blocked for 2 h in 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 (Sigma) or for 1 h in Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE, USA), and incubated overnight with an appropriate dilution of one of the following primary antibodies: (1) phospho-AMPK-alpha (Thr172) antibody (1:1000, Cell Signaling Technology, Inc., Beverly, MA, USA), (2) AMPK alpha antibody (1:1000, Cell Signaling Technology), (3) phospho-acetyl-CoA carboxylase (Ser79) antibody (1:5000, Cell Signaling Technology), (4) acetyl-CoA carboxylase antibody (1:2000, Cell Signaling Technology) or (5) ChREBP polyclonal antibody (1:1000, Cayman Chemical, Ann Arbor, MI, USA). Each of these primary antibodies has previously been validated for use with chicken samples (Proszkowiec-Weglarz et al., 2006a, 2008; Proszkowiec-Weglarz and Richards, 2009). Detection of protein was performed using donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10,000, Amersham Biosciences, Piscataway, NY, USA) with ECL plus Western blotting detection reagents (Amersham) or using an infrared dye-labeled goat polyclonal anti-rabbit IgG (1:20,000, Li-Cor). The membranes were then exposed to Kodak BioMax MR films (Kodak, Rochester, NY, USA), developed, scanned and the band intensities were quantified using densitometry with Imagequant software (Molecular Dynamics, Inc., Sunnyvale, CA, USA). When using the infrared dye-labeled secondary antibody, the membranes were scanned directly after incubation and the band intensities were quantified using the Odyssey infrared imaging system (Li-Cor). Dilutions and incubations with antibodies were performed in Tris-saline buffers containing 0.1% Tween-20 and 1% non-fat dry milk for ECL Plus system or in Odyssey blocking buffer supplemented with 0.1% Tween-20 (primary antibody) or 0.1% Tween-20 and 0.01% SDS (secondary antibody) for the infrared dye system.

Table 2
Sequences of carbohydrate-response-element-containing double-stranded deoxyribonucleic oligonucleotides used in the electrophoretic mobility shift assay (EMSA).

Oligonucleotide	Sequence (5' → 3')
cMPK ChoRE	CTCTGCAAGGGgggttGCCGTGTTGGC
	GCCAACACGGCaacccCCCTTGCAGAG
cS14α ChoRE	CTCAGCACCGCtatagAACGTGGCTGA
	TCAGCCACGTTctataGCGGTGCTGAG
rS14 ChoRE	GTTCTCACGTGgtggcCCTGTGCTTGG
	CCAAGCACAGGgccacCACGTGAGAAC

Oligonucleotide sequences containing ChoRE motifs were designed based on 5000 bp of sequence upstream (5′) to the start codon (ATG) of chicken muscle (*M* type) pyruvate kinase (*cMPK*), chicken *Spot 14α* (cS14α) and rat *Spot 14* (rS14) genes. E-boxes are highlighted in gray while lowercase characters indicate the required 5 bp sequence separating the two E-boxes.

2.7. Enzyme-linked immunosorbent assay (ELISA) for phosphorylated AMPKα

The phosphorylation level of AMPKα in whole liver tissue lysates was determined using the AMPKα [pT172] ELISA kit (Biosource International, Inc., Camarillo, CA, USA) according to the supplier's protocol. Absorbance was measured at 450 nm using a Spectramax M5e plate reader (Molecular Devices, Sunnyvale, CA, USA), and the concentration of phosphorylated AMPKα was calculated from a standard curve (supplied with kit) and normalized to the protein concentration of the lysates (units/100 μg protein).

2.8. Electrophoretic mobility shift assay (EMSA)

Single-stranded (sense and antisense) 5′-infrared dye labeled (IRDye700 or 800) or unlabeled oligonucleotides containing chicken M type pyruvate kinase (cMPK), chicken Spot 14α (cS14α), and rat Spot 14 (rS14) ChoRE sequences (Table 2) were purchased from commercial suppliers (Li-Cor; Invitrogen). Doubled-stranded oligonucleotides were prepared by mixing equal amounts of the complementary single-stranded DNAs, heating to 96 °C for 5 min and slow cooling to room temperature. The binding reaction was performed according to the manufacturer's protocol using the Odyssey infrared EMSA kit (Li-Cor) and 2.5 μg of nuclear extract or 5 μg of whole tissue lysate protein. After 20 min of incubation at room temperature the DNA-protein complexes were separated by electrophoresis on non-denaturating 5% polyacrylamide TBE gels (Bio-Rad) and scanned directly using the Odyssey infrared imaging system (Li-Cor). To confirm specific binding, unlabeled (competitor) oligonucleotides were added at 200- or 4000-fold excess to the reaction.

2.9. Plasma glucose and hormone levels

Plasma glucose was determined using a commercial kit and glucose analyzer (YSI Inc., Yellow Springs, OH, USA). Specific radioimmunoassay (RIA) or ELISA protocols were used to determine plasma hormone concentrations. All samples were analyzed within a single assay to avoid interassay variations. Double-antibody RIA was used to determine plasma concentrations of insulin with an intra-assay coefficient of variation (CV) of 2.2% (McMurtry et al., 1983) and leptin with an intraassay CV of 3.9% (Evock-Clover et al., 2002). Triiodothyronine and T_4 were determined as previously described (McMurtry et al., 1988) with intraassay CVs of 2.5 and 2.8%, respectively. Plasma glucagon was determined as described by McMurtry et al. (1996) using a commercial kit (Linco Research Inc., St. Charles, MO, USA) with an intraassay CV of 1.9%.

2.10. Statistical analysis

Gene (mRNA) and protein expression data were subjected to analysis of variance (ANOVA) using the general linear models (GLM) procedure of SAS (The SAS System for Windows, v. 9.1; SAS Institute, Cary, NC, USA). Duncan's multiple range test was used to determine significance of mean differences. Pearson correlation coefficients and their significance (*P*) values describing the relationships between plasma glucose and hormone levels and the levels of hepatic gene (mRNA) expression were calculated using the CORR procedure of SAS. Statistical significance was set at $P<0.05$.

3. Results

3.1. Plasma glucose and hormone levels during fasting and refeeding

Plasma glucose declined with fasting and was significantly ($P<0.05$) lower than the fed control (C) level at 24 h, but not at 48 h (Table 3). Refeeding for 24 h following a fast significantly ($P<0.05$) increased plasma glucose levels. Plasma levels of insulin, leptin and T_3 decreased significantly ($P<0.05$) compared to the fed control values during a 24 or 48 h fast (Table 3). In contrast, plasma glucagon and T_4 levels increased significantly ($P<0.05$) during fasting (Table 3). Refeeding for 24 h reversed the changes in plasma hormone levels that occurred in response to fasting.

3.2. Activation of hepatic AMPK in response to fasting and refeeding

A modest (45%), but significant ($P<0.05$), increase in phosphorylation (i.e., activation) of the AMPKα subunit (pAMPK) compared to fed control (C) birds was detected by quantitative ELISA after 48 h of fasting in liver (Fig. 1A). This increase in pAMPK was also confirmed by Western blot analysis of the same samples (data not shown). A significant ($P<0.05$) decline in pAMPK occurred with refeeding for 24 h following a prior period of fasting. We next determined the level of phosphorylation of a known target and indicator of AMPK activity, ACC by Western blot (Fig. 1B) and expressed our results as the ratio of phosphorylated ACC (pACC) to total ACC protein (tACC) as an indirect measure of AMPK activity (Fig. 1C). We observed a significant ($P<0.05$) decrease in pACC/tACC only in the group fasted for 24 h and refeeding was without effect on this ratio compared to fed control values (Fig. 1B, C). Moreover, we found that tACC decreased during fasting and increased above the fed control levels upon refeeding indicative of a classical 'overshoot' response (Fig. 1B).

Table 3
Plasma glucose and hormone levels during fasting and refeeding.

	C	S24	S48	S24R24	S48R24
Glucose (mMol/L)	14.65 ± 0.39^{ab}	12.21 ± 0.11^{c}	13.82 ± 0.22^{b}	13.76 ± 0.22^{b}	14.93 ± 0.44^{a}
Insulin (ng/mL)	5.29 ± 0.56^{a}	1.92 ± 0.07^{c}	1.67 ± 0.22^{c}	3.94 ± 0.37^{b}	5.62 ± 0.53^{a}
Glucagon (pg/mL)	131 ± 20^{b}	418 ± 30^{a}	372 ± 31^{a}	176 ± 27^{b}	141 ± 22^{b}
Leptin (ng/mL)	8.85 ± 0.73^{a}	5.49 ± 0.24^{b}	3.30 ± 0.31^{c}	6.41 ± 0.59^{b}	5.89 ± 0.52^{b}
T_3 (ng/mL)	2.28 ± 0.23^{a}	0.81 ± 0.07^{b}	0.99 ± 0.06^{b}	2.47 ± 0.19^{a}	2.12 ± 0.17^{a}
T_4 (ng/mL)	6.43 ± 0.65^{bc}	9.03 ± 0.38^{a}	7.62 ± 0.39^{b}	5.27 ± 0.57^{c}	6.71 ± 0.33^{bc}

Each value represents the mean ± SEM from 6 determinations ($n=6$). Different superscripts in the row indicate that means are significantly different ($P<0.05$).

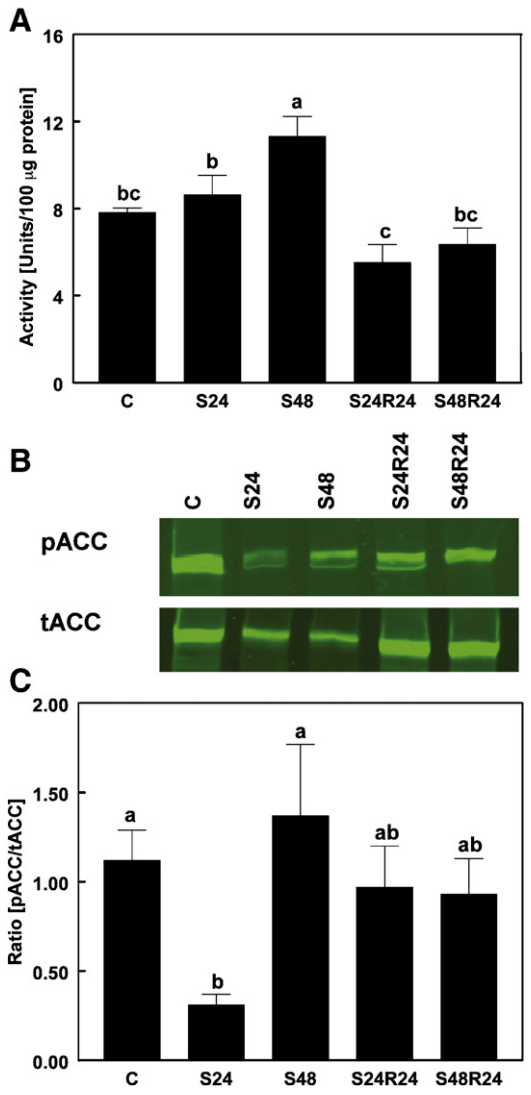

Fig. 1. Effects of fasting and refeeding on the phosphorylation level of AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) in liver tissue from 3-week-old broiler chickens fasted for 24 h (S24) or 48 h (S48) and refed for 24 h after fasting for 24 h or 48 h (S24R24 and S48R24, respectively). Control (C) birds were fed *ad libitum*. (A) Determination of active (phosphorylated) AMPK in liver extracts using an enzyme-linked immunosorbent assay (ELISA) method specific for phosphorylation of threonine 172 contained in the α catalytic subunit of the AMPK complex. Values are expressed as units of AMPK activity (as determined from a standard curve) per 100 μg of protein. (B) Representative Western blot for phospho- and total ACC (pACC and tACC, respectively). (C) Quantitative results from Western blots used to measure pACC and tACC. Values are expressed as the mean ± SEM of the pACC/tACC ratio. Different letters above each bar denote statistically significant ($P<0.05$) differences for mean comparisons.

3.3. Expression of AMPK, ChREBP, SREBP-1 and related genes during fasting and refeeding

Fasting for 24 or 48 h and subsequent refeeding for 24 h had no significant ($P>0.05$) effects on mRNA expression for the two AMPK alpha subunit isoform (α-1 and α-2) genes (data not shown). Also, fasting and refeeding had no significant ($P>0.05$) effects on mRNA expression for *LXR* and *Mlx* genes (Table 4). However, mRNA expression levels for *ChREBP*, *SREBP-1*, and *Spot 14* (α and β) were significantly ($P<0.05$) lower during fasting compared to fed control (C) levels in liver (Table 4). After 24 h of refeeding, mRNA expression for *ChREBP*, *SREBP-1* and *Spot 14* (α and β isoforms) genes returned to fed control levels.

Table 4
Relative mRNA expression of liver transcription factor and co-activator genes during fasting and refeeding.

Gene	C	S24	S48	S24R24	S48R24
LXR	2.63 ± 0.11	2.45 ± 0.08	2.44 ± 0.08	2.59 ± 0.08	2.56 ± 0.08
Mlx	1.13 ± 0.17^{ab}	1.04 ± 0.09^{b}	1.21 ± 0.10^{ab}	1.34 ± 0.06^{a}	1.28 ± 0.04^{ab}
ChREBP	0.69 ± 0.05^{a}	0.59 ± 0.02^{b}	0.54 ± 0.03^{b}	0.68 ± 0.02^{a}	0.74 ± 0.03^{a}
SREBP-1	2.35 ± 0.12^{a}	1.44 ± 0.05^{b}	1.46 ± 0.10^{b}	2.39 ± 0.08^{a}	2.59 ± 0.08^{a}
Spot 14α	3.52 ± 0.09^{a}	1.83 ± 0.11^{b}	1.94 ± 0.17^{b}	3.71 ± 0.10^{a}	3.61 ± 0.13^{a}
Spot 14β	0.23 ± 0.06^{a}	0.02 ± 0.00^{b}	0.02 ± 0.01^{b}	0.33 ± 0.09^{a}	0.36 ± 0.07^{a}

RT–PCR and CE-LIF were used to quantify the level of mRNA expression relative to an 18 S rRNA internal standard. Each value represents the mean ± SEM of 6 determinations ($n=6$). Different superscripts within a row indicate that means are significantly different ($P<0.05$).

3.4. Expression of lipogenic enzyme genes in response to fasting and refeeding

Fasting for 24 or 48 h significantly ($P<0.05$) decreased malic enzyme (ME), ATP-citrate lyase (ACL), ACC, FAS and SCD-1 mRNA levels compared to *ad libitum* fed controls (C), but had no major effect on MCD mRNA expression in liver (Table 5). Refeeding for 24 h restored ACC, ACL, ME, FAS and SCD-1 mRNA expression to the fed control level or, in some cases, increased expression levels significantly ($P<0.05$) above that level indicative of a classical 'overshoot' response (Table 5).

3.5. Correlations between plasma glucose and hormone levels and hepatic gene expression

Table 6 summarizes correlations found between plasma glucose and hormone levels and mRNA expression levels determined for hepatic transcription factor and lipogenic enzyme genes during fasting and refeeding. Expression of mRNA for all lipogenic enzyme genes demonstrated significant ($P<0.01$) and highly positive correlations (>0.8) with each other. These findings suggest a high degree of coordinate regulation for this group of genes involved in lipid synthesis. Expression of mRNA for *ChREBP*, *SREBP*-1, *Spot 14α*, and *LXR* genes each demonstrated significant ($P<0.05$) positive correlations with lipogenic enzyme gene mRNA expression values, as well as with each other. A significant ($P<0.05$) positive correlation was also found between mRNA expression levels of *ChREBP* and *Mlx*. In contrast, *MCD* mRNA levels were found to be negatively correlated with all expressed lipogenic enzyme genes. However, not all of the correlations were found to be significant (e.g., ACCα and FAS). MCD mRNA levels also showed significant ($P<0.05$) negative correlations with *ChREBP*, *SREBP-1* and *Spot 14α* mRNA expression, but were not significantly correlated with the mRNA levels for *LXR* or *Mlx*. Moreover, *MCD* mRNA levels demonstrated significant ($P<0.05$) negative correlations with plasma insulin and leptin levels and a significant ($P<0.05$) positive correlation with glucagon. Plasma levels of insulin, T_3 and leptin showed significant ($P<0.05$) positive correlations with *ChREBP*, *SREBP-1*, *Spot 14α*, and lipogenic enzyme mRNA

Table 5
Relative mRNA expression of liver lipogenic enzyme genes during fasting and refeeding.

Gene	C	S24	S48	S24R24	S48R24
ME	2.69 ± 0.13^{c}	1.50 ± 0.06^{d}	1.42 ± 0.08^{d}	3.17 ± 0.14^{b}	3.66 ± 0.07^{a}
ACL	2.81 ± 0.10^{b}	2.27 ± 0.09^{c}	2.21 ± 0.05^{c}	2.88 ± 0.05^{b}	3.23 ± 0.06^{a}
ACC	2.43 ± 0.19^{b}	1.85 ± 0.12^{c}	1.794 ± 0.16^{c}	3.32 ± 0.052^{a}	3.30 ± 0.13^{a}
MCD	1.64 ± 0.07^{b}	1.89 ± 0.13^{a}	1.83 ± 0.05^{ab}	1.71 ± 0.08^{ab}	1.79 ± 0.08^{ab}
FAS	2.60 ± 0.17^{b}	1.21 ± 0.09^{c}	1.24 ± 0.14^{c}	3.00 ± 0.27^{b}	3.64 ± 0.20^{a}
SCD-1	2.55 ± 0.16^{b}	0.52 ± 0.03^{c}	0.52 ± 0.04^{c}	2.96 ± 0.06^{a}	3.02 ± 0.07^{a}

RT–PCR and CE-LIF were used to quantify the level of mRNA expression relative to an 18 S rRNA internal standard. Each value represents the mean ± SEM of 6 determinations ($n=6$). Different superscripts within a row indicate that means are significantly different ($P<0.05$).

Table 6
Correlations between plasma glucose and hormone levels and expression of hepatic transcription factor and lipogenic enzyme genes.*

	Glucose	Insulin	Glucagon	Leptin	T_3	T_4	*CHREBP*	*SREBP-1*	*Spot 14α*	*LXR*	*MLX*	*ME*	*ACL*	*ACC*	*FAS*	*SCD-1*	*MCD*
Glucose	1.00000	**0.61376**	**−0.61974**	0.30807	**0.49396**	**−0.40742**	**0.46902**	**0.59910**	**0.57335**	0.22342	0.27191	**0.58160**	**0.61401**	**0.51652**	**0.56518**	**0.56869**	**−0.42493**
		0.00030	0.00030	0.09770	0.00550	0.02540	0.01030	0.00050	0.00090	0.23530	0.15360	0.00070	0.00030	0.00350	0.00110	0.00100	0.01920
Insulin		1.00000	**−0.76670**	**0.60710**	**0.70882**	**−0.45728**	**0.65176**	**0.75062**	**0.75441**	0.22547	0.27254	**0.75669**	**0.78294**	**0.62503**	**0.68027**	**0.79390**	**−0.36755**
			<.0001	0.00040	<.0001	0.01110	0.00010	<.0001	<.0001	0.23090	0.15260	<.0001	<.0001	0.00020	<.0001	<.0001	0.04570
Glucagon			1.00000	**−0.55991**	**−0.76596**	**0.59922**	**−0.67680**	**−0.84660**	**−0.84540**	**−0.40165**	−0.27458	**−0.82292**	**−0.78140**	**−0.63130**	**−0.75171**	**−0.87679**	**0.49206**
				0.00130	<.0001	0.00050	<.0001	<.0001	<.0001	0.02780	0.14940	<.0001	<.0001	0.00020	<.0001	<.0001	0.00570
Leptin				1.00000	**0.49483**	−0.20907	**0.48650**	**0.59731**	**0.58451**	**0.40577**	0.01175	**0.45722**	**0.51888**	0.35843	**0.41489**	**0.55160**	**−0.42879**
					0.00540	0.26750	0.00740	0.00050	0.00070	0.02610	0.95180	0.01110	0.00330	0.05180	0.02260	0.00160	0.01810
T_3					1.00000	**−0.70508**	**0.61341**	**0.76602**	**0.81483**	0.28200	0.15268	**0.75576**	**0.67399**	**0.63353**	**0.76263**	**0.85936**	−0.25028
						<.0001	0.00040	<.0001	<.0001	0.13110	0.42910	<.0001	<.0001	0.00020	<.0001	<.0001	0.18220
T_4						1.00000	**−0.56903**	**−0.56924**	**−0.63614**	−0.15787	**−0.45346**	**−0.60755**	**−0.53764**	**−0.53809**	**−0.59685**	**−0.67903**	0.21518
							0.00130	0.00100	0.00020	0.40470	0.01350	0.00040	0.00220	0.00220	0.00050	<.0001	0.25350
CHREBP							1.00000	**0.81935**	**0.75166**	**0.62137**	**0.47273**	**0.81025**	**0.86147**	**0.56080**	**0.67351**	**0.77335**	**−0.37096**
								<.0001	<.0001	0.00030	0.00960	<.0001	<.0001	0.00160	<.0001	<.0001	0.04760
SREBP-1								1.00000	**0.94059**	**0.56003**	0.29861	**0.94183**	**0.93546**	**0.74181**	**0.83208**	**0.94434**	**−0.43205**
									<.0001	0.00130	0.11560	<.0001	<.0001	<.0001	<.0001	<.0001	0.01710
Spot 14α									1.00000	**0.46926**	0.30750	**0.91114**	**0.85852**	**0.73376**	**0.81892**	**0.95276**	**−0.46853**
										0.00890	0.10470	<.0001	<.0001	<.0001	<.0001	<.0001	0.00900
LXR										1.00000	**0.36868**	**0.43446**	**0.48423**	0.19344	0.23318	**0.40536**	−0.26520
											0.04910	0.01640	0.00670	0.30570	0.21490	0.02630	0.15670
MLX											1.00000	**0.37063**	**0.42090**	**0.41900**	0.28912	0.33254	−0.05108
												0.04780	0.02300	0.02370	0.12820	0.07800	0.79240
ME												1.00000	**0.95824**	**0.84260**	**0.88638**	**0.96171**	−0.33576
													<.0001	<.0001	<.0001	<.0001	0.06970
ACL													1.00000	**0.81504**	**0.82869**	**0.91039**	**−0.36883**
														<.0001	<.0001	<.0001	0.04490
ACC														1.00000	**0.80898**	**0.82742**	−0.26906
															<.0001	<.0001	0.15050
FAS															1.00000	**0.88407**	−0.20835
																<.0001	0.26920
SCD-1																1.00000	**−0.41747**
																	0.02170
MCD																	1.00000

*Values include Pearson correlation coefficients with *P* values and coefficients found to be significant ($P<0.05$) are shown in bold typeface.

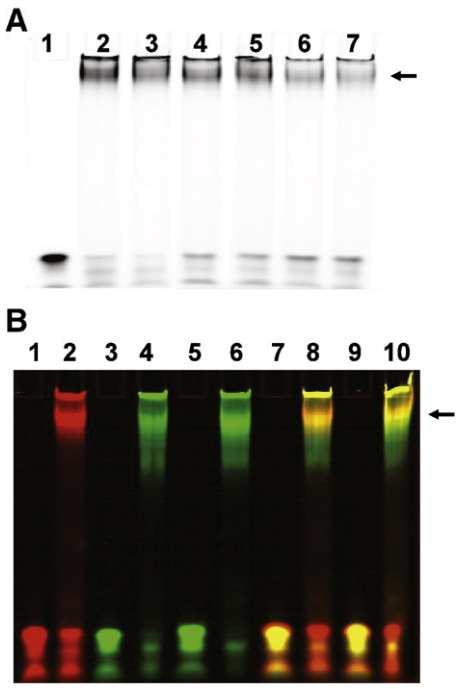

Fig. 2. (A) Electrophoretic mobility gel shift assay (EMSA) analysis showing the binding of chicken liver nuclear protein extracts to an infrared dye-labeled oligonucleotide (double-stranded DNA) containing the carbohydrate response element (ChoRE) from the chicken muscle pyruvate kinase (*cMPK*) gene promoter (see Table 2 for oligonucleotide sequences). Chickens were fed *ad libitum* (lane 2, 4 and 6) or fasted for 24 h (lane 3, 5 and 7). Additionally, samples in lanes 4–5 and 6–7 were incubated with an excess of unlabeled oligonucleotide (200× and 4000×, respectively) as a competitor to demonstrate specificity in the binding. Lane 1 depicts a sample containing only the infrared dye-labeled oligonucleotide. The arrow indicates the position of the shifted ChREBP/DNA binding complex. (B) Binding comparison for whole liver tissue protein extracts to infrared dye-labeled oligonucleotides (double-stranded DNA) containing the ChoRE from the cMPK, chicken *Spot 14* alpha (cS14), and rat *Spot 14* (rS14) gene promoters (see Table 2 for the oligonucleotide sequences). The cMPK ChoRE-containing oligonucleotide was labeled with infrared dye 700 (IRDye700) while the cS14 ChoRE- and rS14 ChoRE-containing oligonucleotides were labeled with IRDye800. EMSA analyses were performed using 5 μg of whole liver tissue protein extract from fed control chickens. The binding specificity was determined using Odyssey two-color imaging software (IRDye700 — green and IRDye800 — red for singleplex reactions) and the yellow color indicates that chicken liver protein binds to the cMPK ChoRE- as well as to cS14ChoRE- or rS14ChoRE-containing oligonucleotides when analyzed in duplex EMSA reactions. Lanes are identified as follows: 1 — cMPK ChoRE; 2 — cMPK ChoRE and liver protein; 3 — cS14 ChoRE; 4 — cS14 ChoRE and liver protein; 5 — rS14 ChoRE; 6 — rS14 ChoRE and liver protein; 7 — cMPK ChoRE and cS14 ChoRE; 8 — cMPK ChoRE, cS14 ChoRE and liver protein; 9 — cMPK ChoRE and rS14 ChoRE; 10 — cMPK ChoRE, rS14 ChoRE and liver protein. The arrow indicates the position of the shifted ChREBP/DNA binding complex.

expression values, whereas glucagon and T_4 were both found to be negatively correlated. Expression of *ChREBP*, *SREBP-1*, *Spot 14α* and each of the lipogenic enzymes all demonstrated significant ($P<0.05$) positive correlations with plasma glucose, whereas *MCD* mRNA levels were negatively correlated. The mRNA expression of *Mlx* showed a significant ($P<0.05$) negative correlation with T_4, whereas *LXR* mRNA levels showed significant ($P<0.05$) negative and positive correlations with glucagon and leptin, respectively.

3.6. DNA-binding activity of hepatic ChREBP

Infrared dye-labeled double-stranded oligonucleotides (50 nM) containing the ChoRE site from the *cMPK* gene promoter (Table 2) were used to verify ChREBP DNA-binding activity in fresh liver nuclear extracts obtained from chickens fed *ad libitum* or fasted for 24 h. Electrophoretic mobility shift assay (EMSA) revealed the ability of nuclear extracts to shift the dye-labeled oligonucleotides and indicated the presence of the DNA-bound ChREBP/Mlx complex (Fig. 2A). This ChREBP DNA-binding activity was lower in extracts from fasted birds (lane 3) compared to those fed *ad libitum* (lane 2). The addition of unlabeled double-stranded cMPK ChoRE-containing oligonucleotides at 200- (10 μM) and 4000- (200 μM) fold excess decreased the DNA-binding activity and demonstrated specificity of the reaction for the ChREBP/MLX complex (Fig. 2A, lanes 4–5 and 6–7, respectively).

To examine the ability of chicken ChREBP protein to bind to other ChoRE sites, dye-labeled double-stranded oligonucleotides (50 nM) containing predicted ChoRE sites from *cS14α* and *rS14* gene promoters (Table 2) were incubated with whole liver tissue lysates obtained from *ad libitum* fed chickens (Fig. 2B). EMSA analysis indicated that chicken ChREBP bound not only to the cMPK ChoRE (lane 2), but also to the cS14α ChoRE (lane 4) as well as to the rS14 ChoRE (lane 6). Chicken

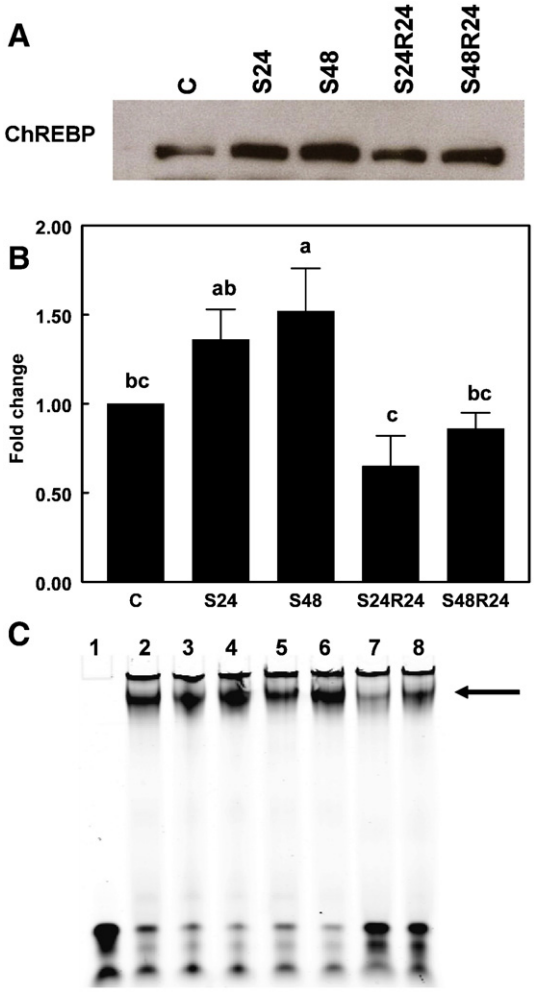

Fig. 3. Effects of fasting and refeeding on ChREBP protein level and its activity in liver tissue from 3-week-old broiler chickens fasted for 24 h (S24) or 48 h (S48) and refed for 24 h after fasting for 24 or 48 h (S24R24 and S48R24, respectively). Control (C) birds were fed *ad libitum*. (A) Representative Western blot for ChREBP protein. (B) Results from Western blots of ChREBP used to quantify protein level. Values (mean ± SEM) are expressed as fold change relative to the control group. Different letters above each bar denote statistically significant ($P<0.05$) differences for mean comparisons. (C) Electrophoretic mobility gel shift assay (EMSA) analysis of the binding of chicken liver protein extracts to an infrared dye-labeled oligonucleotide (double-stranded DNA) containing the carbohydrate response element (ChoRE) from the chicken muscle pyruvate kinase (*cMPK*) gene promoter. Chickens were fed *ad libitum* (lane 2, 7), fasted for 24 h (lane 3) or 48 h (lane 4), and refed for 24 h after fasting for 24 h (lane 5) or 48 h (lane 6, 8). Additionally samples in lanes 7 and 8 were incubated with an excess (4000×) of unlabeled oligonucleotide as a competitor. Lane 1 depicts a sample containing only the infrared dye-labeled oligonucleotide. The arrow indicates the position of the shifted ChREBP/DNA binding complex.

ChREBP appeared to bind with higher affinity to the rS14 ChoRE than to cMPK ChoRE or cS14α ChoRE (lanes 6, 2 and 4, respectively). However, when two individual ChoRE-containing oligonucleotides (each with different IRDye labels) were incubated together (lane 8: cMPK ChoRE and cS14α ChoRE; lane 10: cMPK ChoRE and rS14 ChoRE), no apparent competition for chicken ChREBP occurred between these sets of oligonucleotides. Thus, it appears that the chicken ChREBP/Mlx complex exhibits no binding preference for different ChoRE sites, at least *in vitro* (Fig. 2B).

3.7. Hepatic ChREBP protein and DNA-binding activity in response to fasting and refeeding

Fasting for 24 or 48 h increased ChREBP protein levels in liver and after 48 h ChREBP protein expression was significantly ($P<0.05$) higher than in livers from fed control birds (Fig. 3A,B). Refeeding for 24 h following a 24 or 48 h fast reduced ChREBP protein to the fed control level. Fig. 3C presents the EMSA analysis of ChREBP DNA-binding activity (cMPK ChoRE) which increased during 24–48 h of fasting (lanes 3 and 4) and declined with subsequent refeeding (lanes 5 and 6). As observed previously, this DNA-binding activity could be specifically competed with the addition of an excess (200- or 4000-fold) amount of unlabeled double-stranded oligonucleotide containing the cMPK ChoRE site (Fig. 3C lanes 7 and 8).

4. Discussion

Hepatic fatty acid synthesis is known to be regulated at the transcriptional level in mammals by changes in nutrient and hormone levels such as those which occur during fasting and refeeding under normal physiological conditions (Desvergne et al., 2006). However, the mechanisms involved are not well understood in birds. In this study we investigated associations between expression of hepatic lipogenic enzyme genes; plasma glucose and metabolic hormone levels; expression of specific transcription factors involved in regulating hepatic lipogenic enzyme genes; and AMPK in broiler chickens to gain insight into underlying mechanisms involved in nutritional and hormonal regulation of the avian hepatic lipogenic program.

4.1. Lipogenic enzyme gene expression

Our data showed very high correlations among mRNA expression values for the major lipogenic enzyme genes in liver which decreased during fasting and increased upon refeeding. Moreover, there was a significant 'overshoot' observed for the expression of these genes in response to refeeding following a 24 or 48 h fast. Transcription of lipogenic enzyme genes is known to be regulated coordinately in response to nutrient and hormonal signals in mammals (Jump et al., 1994; Towle et al., 1996, 1997), and this has also been suggested to occur in birds (Daval et al., 2000; Assaf et al., 2003). Recently, Desert et al. (2008) utilized chicken 20 K oligonucleotide (pangenomic) microarrays to perform global transcriptional profiling of liver. They demonstrated coordinate down-regulation of various lipogenic genes (*ACL*, *FAS*, *ACCα* and *SCD-1*) occurred in 4-week-old broiler chickens subjected to fasting for 16 or 48 h. The high degree of correlation observed among individual lipogenic enzyme gene mRNA expression patterns in fasted and fed states clearly indicates that lipogenesis in chicken liver is tightly and coordinately regulated at the transcriptional level.

Nutritional regulation of hepatic lipogenic activity in chickens has previously been shown to occur in response to changes in the energy to protein ratio of the diet or by repeated cycles of fasting and refeeding (Leveille et al., 1975; Rosebrough, 2000; Rosebrough et al., 2002). An increase above fed control levels during refeeding (i.e., 'overshoot') has been observed previously in broiler chicken liver with respect to lipogenic enzyme activities and *in vitro* lipogenesis measurements, becoming more pronounced with repeated cycles of fasting and refeeding (Rosebrough, 2000). Feed restriction has also been shown to significantly alter the expression of key genes involved in hepatic lipogenesis in broiler breeder chickens (Richards et al., 2003; de Beer et al., 2007). Rosebrough (2000) suggested that the decrease observed in hepatic lipogenesis in response to fasting or feeding high protein diets to broiler chickens was likely due to reduced flow of carbon-based substrates through the glycolytic pathway and increased production of glucose (via gluconeogenesis) from those substrates that would otherwise be utilized for lipid synthesis. Thus, hepatic lipid synthesis is stringently regulated in response to changes in energy status by controlling carbon flux through the glycolytic and lipogenic pathways with expression of the rate-limiting glycolytic and lipogenic enzymes controlled, in part, at the transcriptional level (Desvergne et al., 2006).

4.2. Hormonal regulation of hepatic lipogenic enzyme gene expression

Insulin and T_3 are two important metabolic hormones known to play a synergistic role in the induction of hepatic lipogenic enzyme gene expression in chicken hepatocytes in culture, whereas glucagon was shown to be antagonistic (Hillgartner et al., 1995). Our results showing a significant positive correlation between the plasma levels of insulin and T_3 coupled with a significant negative correlation between plasma glucagon and individual hepatic lipogenic enzyme gene expression patterns is consistent with such hormonal regulation of lipogenesis *in vivo*. We also observed a significant positive association between plasma leptin level and hepatic lipogenic enzyme mRNA expression. Leptin administration has recently been reported to significantly induce the expression of FAS and SCD-1 in the liver of broiler chickens, even in fasted birds or birds administered cerulenin which inhibits food intake (Dridi et al., 2005, 2007). In a previous study, we found that leptin administered to fed, but not fasted, broiler chickens induced a significant increase in plasma triglycerides (Evock-Clover et al., 2002). Together, these observations suggest that leptin may stimulate hepatic lipogenesis in birds. This is in sharp contrast to the inhibitory effects of leptin on hepatic SCD-1 expression reported in mice (Biddinger et al., 2006). Studies in mammalian animal and cell culture models suggested that the central nervous system (CNS) as opposed to the liver is the major site of leptin action on hepatic *SCD-1* gene expression (Biddinger et al., 2006). These differences may reflect species- and/or tissue-specific effects of leptin on hepatic lipogenic enzyme gene expression in birds.

4.3. Transcription factor-mediated regulation of the lipogenic program

Transcriptional regulation of hepatic lipogenesis in chickens by a common set of regulatory factors is suggested by the high degree of correlation observed in this study between the mRNA expression levels of certain transcription factors and coregulators (i.e., SREBP-1, ChREBP and Spot 14) and the expression of individual lipogenic enzyme genes which have been reported to be targets for these factors. However, it was not possible to distinguish between direct and indirect effects because of complex interrelationships among the different regulatory factors and transcriptional networks potentially involved (see Fig. 4).

A single LXR protein, similar to the LXRα isoform expressed in mammals, is expressed in birds and it may play a role in regulating hepatic lipogenesis (Proszkowiec-Weglarz et al., 2008; Han et al., 2009). The lack of significant correlation between mRNA expression patterns of LXR and some of its lipogenic enzyme target genes such as ACCα and FAS observed in our study most likely indicates that some of the effects of LXR on lipogenic gene transcription are mediated by post-transcriptional events such as the binding of and activation by lipid ligands (e.g., oxysterols) and not by increased transcription of the *LXR* gene. Han et al. (2009) found that treating primary goose hepatocytes in culture with the LXRα agonist TO-901317 increased mRNA expression of SREBP-1, FAS, ACCα as well as LXR itself in a dose-dependent manner. The latter finding could indicate the existence of a

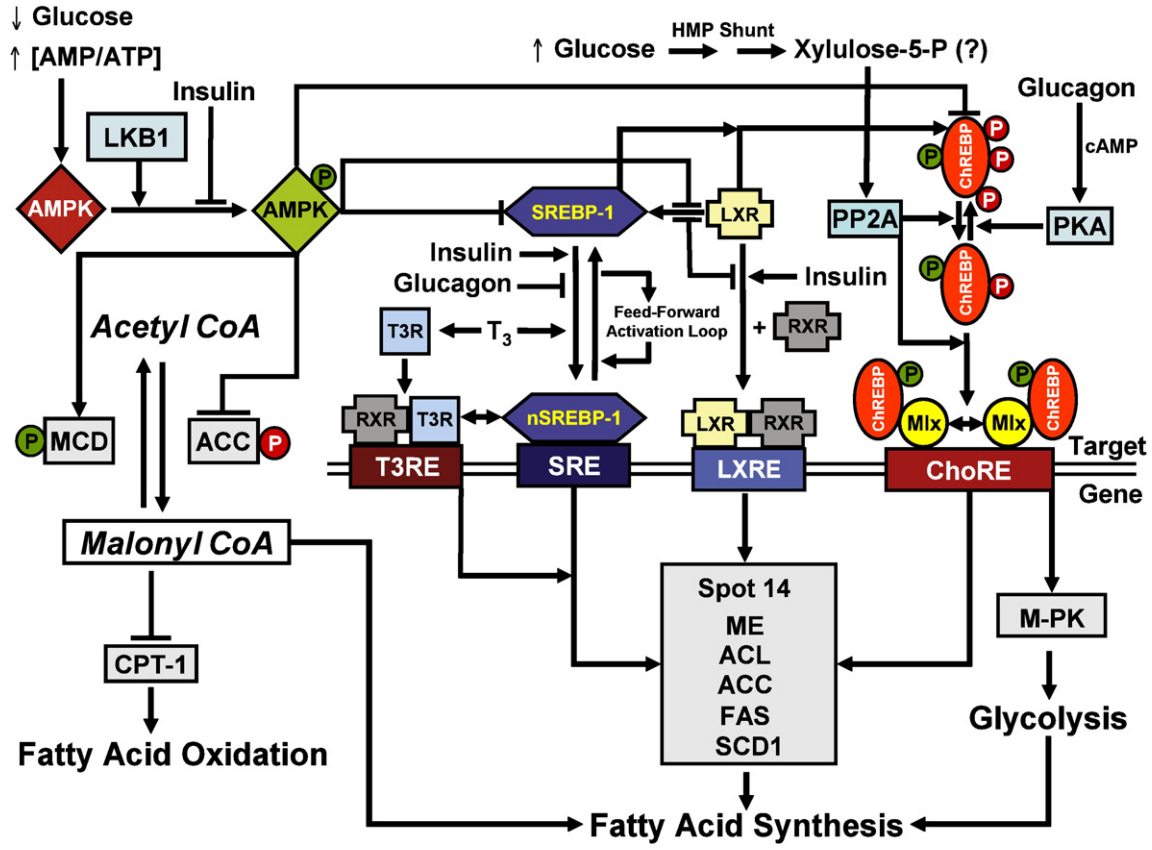

Fig. 4. A proposed model depicting potential roles of AMP-activated protein kinase (AMPK), carbohydrate response element binding protein (ChREBP) and their relationships with other factors which integrated together form critical control points for the regulation of hepatic lipogenic gene expression in birds. Arrows (↓) indicate stimulatory pathways while capped lines (⊥) indicate inhibitory effects. Stimulatory or inhibitory effects of protein phosphorylation are indicated by green or red circles, respectively.

feed-forward regulatory loop for the *LXR* gene in birds; however, that remains to be demonstrated experimentally. Despite the lack of any significant effects of fasting and refeeding, we found that LXR mRNA expression was positively correlated with the expression of ChREBP, SREBP-1 and Spot 14 (Table 6). This could indicate a cooperative or indirect role for LXR in mediating increased transcription of lipogenic enzyme genes in birds in addition to any direct effects it might have. Indeed, mice lacking the *LXRα* gene exhibit lower basal levels of SREBP-1c protein expression in addition to reduced mRNA levels for ACCα, FAS and SCD-1 (Schultz et al., 2000).

We observed a decrease in hepatic SREBP-1 mRNA expression with fasting and a subsequent increase upon refeeding. This pattern closely followed that observed for the expression of individual lipogenic enzyme genes. Desert et al. (2008) also observed significant positive correlations between the expression of SREBP-1 and lipogenic enzyme genes in chicken liver during fasting. However, no 'overshoot' was observed in our study for SREBP-1 mRNA levels upon refeeding as was found for the lipogenic enzyme genes. Bennett et al. (2008) recently reported that the lipogenic enzyme gene expression 'overshoot', observed in response to refeeding a high carbohydrate diet to mice following a fast, was attributable to enhanced SREBP-1c binding to SRE sites located in lipogenic enzyme target gene promoters as well as increased coregulatory protein recruitment. Zhang and Hillgartner (2004) reported that feeding a high carbohydrate, low fat diet to broiler chickens following a fast markedly stimulated the concentration and binding activity (to the ACCα promoter) of SREBP-1 protein in liver.

We found significant positive correlations between SREBP-1 mRNA expression in liver and plasma insulin and T_3 levels, whereas glucagon exhibited a strong negative correlation. In mammals, SREBP-1c acts as a master regulator of lipogenesis in the liver and serves as the major mediator for insulin-induced hepatic lipogenic gene expression (Horton et al., 2002). Birds, only express a single isoform of SREBP-1, the level of which is closely linked with lipogenic enzyme gene transcription (Zhang et al., 2003). Using immuno-neutralization, Dupont et al. (2008) showed that insulin deprivation in young growing broiler chickens mimicked fasting conditions by reducing mRNA and protein expression for SREBP-1 in liver concomitant with a reduction in mRNA expression for FAS. Zhang and Hillgartner (2004) showed that T_3 and insulin increased SREBP-1 expression in liver via pre- and post-translational mechanisms, respectively. This group also showed that SREBP-1 interacts with the nuclear thyroid hormone receptor (T3R) forming a complex along with RXR and interacting with SRE and thyroid response element (T3RE) promoter sites to enhance ACCα gene transcription in chick embryo hepatocytes (Yin et al., 2002). In contrast, cAMP (and presumably glucagon which stimulates cAMP production) suppressed SREBP-1 in chick embryo hepatocytes via a post-translational mechanism (Zhang et al., 2003). Together, these findings suggest a central role for SREBP-1 in mediating the effects of fasting and refeeding on lipogenic enzyme gene transcription in the liver of birds by integrating the actions of insulin, T_3 and glucagon (Zhang et al., 2003). However, differences in the number of SREBP-1 isoforms and the mechanisms mediating the effects of insulin on mature SREBP-1 protein levels may reflect a modified role for SREBP-1 in regulating hepatic lipogenesis in birds compared to mammals (Zhang et al., 2003).

The expression of Spot 14 mRNA showed significant correlations with plasma T_3, glucose, insulin and glucagon, all of which are known to affect the expression of this lipogenic gene-associated potential coregulator. Moreover, the pattern of changes in Spot 14 mRNA levels mimicked each of the lipogenic enzyme genes during fasting and

refeeding (with the exception of the refeeding-induced 'overshoot') suggesting a coordinate regulatory mechanism(s) governing the expression of these genes. Two *Spot 14* genes (i.e., α and β isoforms) have been identified in birds, with *Spot 14α* being the predominant isoform gene expressed in liver (Wang et al., 2004; Zhan et al., 2006; Proszkowiec-Weglarz et al., 2008). Our findings suggest that *Spot 14* in birds could play an important role in integrating nutrient and hormone signals in the regulation of hepatic lipogenesis. However, this remains to be determined experimentally.

The fact that plasma glucose showed significant and positive correlations with mRNA expression of lipogenic enzyme genes in this study suggests that glucose could play a direct role in regulating hepatic lipogenesis in birds as it does in mammals. However, plasma insulin, T_3 and glucagon were more highly correlated with hepatic lipogenic enzyme gene expression than glucose which suggests that these hormones might play a more prominent role in regulating hepatic lipogenesis in birds than glucose. Hillgartner and Charron (1998) reported that glucose stimulated transcription of *FAS* and *ME* genes in chick embryo hepatocytes. They also showed that this stimulation involved a metabolite of the pentose phosphate pathway and required the presence of T_3, but not insulin. In light of these findings, it was concluded that the mechanism by which glucose regulates transcription of hepatic lipogenic enzyme genes in birds differs from that in mammals which do not require the presence of T_3. Dentin et al. (2004) previously determined that, in mammals, the action of glucose on lipogenic gene expression required GK and glucose metabolism (i.e., G6P production) for maximal induction. Lower hepatic activities of GK and the pentose pathway observed in chickens (Goodridge, 1968; Rideau et al., 2008) suggest that glucose signaling might play a different (i.e., lesser?) role in regulating hepatic lipogenesis in birds than it does in mammals.

In mammals, glucose-dependent regulation of glycolytic and lipogenic enzyme gene transcription requires the actions of ChREBP and its coactivator Mlx (Ma et al., 2005). We have previously identified and characterized *ChREBP* and *Mlx* genes in the chicken and demonstrated the presence of ChoREs in the promoters of *M-PK*, ACCα and *Spot 14α* genes, as well as the *ChREBP* gene itself, indicating their potential for glucose-mediated transcriptional regulation via ChREBP/Mlx (Proszkowiec-Weglarz et al., 2008). In this study we observed a significant positive correlation between plasma glucose and ChREBP mRNA expression and between the expression of ChREBP and Mlx, consistent with the existence of a feed-forward transcriptional regulatory loop. However, relatively low correlations between Mlx expression and some of the lipogenic enzyme genes (*ME*, *ACL*, ACCα) were observed. Such observations do not necessarily diminish the importance of this transcriptional coregulator in the glucose-dependent regulation of hepatic lipogenic gene expression in birds. In fact, it has recently been reported that hepatic expression of a dominant negative *Mlx* gene construct in mice inhibited expression of glucose-regulated genes including *ChREBP* (Iizuka et al., 2009).

While the mechanisms controlling ChREBP mRNA expression and protein activation have been well characterized in mammals (Uyeda and Repa, 2006; Postic et al., 2007), most of the studies have been conducted in cultured cells using extreme levels (both high and low) of glucose added to the media. The effects of nutritional and hormonal control of ChREBP expression and activity *in vivo* under normal physiological conditions have received considerably less attention (Letexier et al., 2005). Intracellular localization (cytoplasmic vs. nuclear) and phosphorylation state of ChREBP are key factors determining its activity. In this study, fasting, accompanied by a modest reduction in plasma glucose, resulted in decreased binding activity of ChREBP in nuclear extracts as assessed by EMSA (Fig. 2). However, whole liver tissue lysates exhibited increased ChREBP protein level and DNA-binding activity in response to fasting which declined upon refeeding (Fig. 3). The latter observations appear to be contrary to what would have been expected considering the observed reduction in plasma glucose and ChREBP mRNA expression in response to fasting. Given the recognized differences in glucose metabolism in birds as compared to mammals, such discrepancies may reflect unique aspects of expression and activation of ChREBP in avian liver. Moreover, our results like those of Letexier et al. (2005) suggest that *in vivo* measurements of ChREBP mRNA and protein expression as well as its DNA-binding activity may not correspond with findings from *in vitro* model systems. Clearly, more investigation is required to evaluate ChREBP expression and activation *in vivo* to better define its role in mediating glucose-dependent regulation of hepatic lipogenesis in birds.

4.4. A role for AMPK in the regulation of hepatic lipogenesis in vivo?

Previous studies in rats reported a strong increase in AMPK activity in liver in response to short-term (i.e., 6 h) fasting accompanied by a rapid reduction in expressed ACC enzymatic activity in conjunction with increased phosphorylation of the protein at a serine (S79) residue exclusively targeted by AMPK (Munday et al., 1991; Witters et al., 1994). These effects were quickly reversed upon refeeding for 2–6 h. This suggested a direct relationship between AMPK activation status and fatty acid synthesis in the liver in response to acute changes in energy status. However, Gonzalez et al. (2004) found no significant activation of AMPK in the liver of rats subjected to a 24 h fast or to chronic (4 months) caloric restriction and suggested that the hypoglycemia induced by a 24 h fast was not severe enough to cause a decline in the liver AMP/ATP ratio of sufficient magnitude to activate AMPK. Moreover, they were also unable to show an effect of fasting on the level of phosphorylated ACC; although, like our study, they did find a dramatic decline in hepatic ACC protein levels during food deprivation. They concluded that while the activation of AMPK plays an important role in metabolic adaptation of cultured cells to acute nutritional stresses such as glucose deprivation *in vitro*, AMPK is not activated by the metabolic challenges of fasting or caloric restriction *in vivo*. We found a modest increase in phosphorylated AMPK in liver after prolonged (48 h) fasting but with no commensurate increase in the phosphorylation state of ACC suggesting the lack of a direct relationship between AMPK activation and regulation of hepatic lipogenesis in birds. We cannot rule out a potential indirect role for AMPK in long-term regulation hepatic lipogenesis in birds via its ability to modulate the actions of transcription factors such as SREBP-1, LXR and ChREBP through post-translational mechanisms (Viollet et al., 2009; Yang et al., 2009); but this has not yet been shown to occur in birds. Desert et al. (2008) suggested that chickens are more resistant to longer periods of food deprivation than rodents and that the severity of a 48 h fast is limited in birds as compared to other species. This may be linked to an enhanced ability of birds to maintain plasma glucose levels during fasting. The modest decline in plasma glucose (17% at 24 h) induced by fasting in this study may not have caused enough of a change in the hepatic cellular AMP/ATP ratio to activate AMPK sufficiently for it to have a sustained impact on the phosphorylation state of ACC. Thus, the dramatic changes we observed in ACC protein levels are more likely to account for the temporal changes in hepatic lipogenesis that have previously been reported to accompany fasting and refeeding of broiler chickens (Rosebrough, 2000). One could reasonably argue that any effect of AMPK on ACC phosphorylation was too transient to be detected within our sampling timeframe. However, Dupont et al. (2008) found no effect of insulin deprivation or a short-term (5 h) fast on AMPK phosphorylation in liver tissue from broiler chickens. We can also confirm the lack of significant change in AMPK and ACC phosphorylation states in response to acute periods (2–6 h) of fasting and refeeding of 3-week-old broiler chickens unpublished findings. Finally, other factors might modulate phosphorylation of ACC by AMPK *in vivo* and thereby regulate the AMPK/ACC pathway in birds. Insulin and glucose have been reported to acutely increase malonyl-CoA levels in liver by dephosphorylating and activating ACC (Ruderman et al., 1999). Cesquini et al. (2008) recently reported that the intermediate

metabolite citrate, an allosteric activator of ACC, decreased AMPK and ACC phosphorylation in liver. The involvement of these or perhaps other factors in regulating the hepatic AMPK/ACC pathway in birds has not yet been explored.

4.5. Conclusions

Individual transcription factors such as SREBP-1 and LXR have been shown to have unique roles in the regulation of the hepatic lipogenic program in birds. However, they do not act alone to control lipogenic gene transcription since there is a high degree of integration among and considerable crosstalk between hepatic transcriptional regulatory networks (Fig. 4). This is due, in part, to the presence of multiple cis-acting elements present in the promoters of key transcription factor and target genes. A wide variety of energy status signals including: glucose, fatty acids, insulin, leptin, glucagon and T_3 working through specific receptors act directly on lipogenic gene expression and indirectly through the actions of specific signal transduction pathways and individual transcription factors. The energy sensor and metabolic transducer AMPK may serve as an integrator of these and other signals to influence lipid metabolism in the liver of birds, although the mechanisms, whether direct or indirect, underlying such a role remain to be defined. Clearly, a highly integrated regulatory system accounts for the coordinated transcriptional control that is observed for the hepatic lipogenic program in birds. The findings from this study using broiler chickens provide further insight into possible mechanisms involved in regulating hepatic lipogenesis *in vivo* commensurate with changes in whole body energy status. While glucose and metabolic hormones are undoubtedly critical factors involved in signaling metabolic adaptation to changing energy status, further studies are needed to better define the specific roles of AMPK and ChREBP in regulating hepatic lipid metabolism *in vivo*.

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